BRIEF COMMUNICATION

DEVELOPMENT OF 5-DAY RABBIT BLASTOCYSTS
AFTER CULTURE AT 37° C

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Summary. Rabbit blastocysts were cultured *in vitro* at 37° C for 8, 16 or 24 hr in modified F₁₀ medium. Subsequent transfer to suitable recipient does revealed that blastocyst viability was maintained *in vitro* for at least 16 hr. The percentage of viable foetuses developing from blastocysts cultured for at least 8 hr was equal to that from non-cultured blastocysts.

The rabbit ovum readily cleaves to the late morula or early blastocysts stage upon culture, but difficulty has been experienced in obtaining further development *in vitro*. The cultured zygotes remain viable and come to term if transferred into host uteri. Expanded blastocysts have been cultured *in vitro* at approximately 37° C with some success (Brachet, 1912; Waterman, 1932; Pincus & Werthessen, 1938; Chang, 1950; Lutwak-Mann, Hay & Adams, 1962), but their successful development following transfer has not been reported.

However, 5-day rabbit blastocysts will increase in size (Daniel, 1965) when cultured at 37° C in a modification of the cell culture medium F₁₀ of Ham (1963). The increase occurred after both 4 and 24 hr in modified F₁₀ alone, but added serum was required to match the size range attained *in vivo*. The purpose of this investigation was to culture such blastocysts *in vitro* at 37° C, in the modified F₁₀ medium with and without serum added, and to test the capacity of the cultured blastocysts to develop into normal foetuses.

Mature, Dutch-Belted does weighing more than 1.75 kg were used (Staples & Holtkamp, 1966). One hundred and eleven virgin does were utilized as blastocyst donors: fifty-eight post-partum does served as recipients of control and cultured blastocysts.

Donor females received an ovulating dose of luteinizing hormone (LH) and were artificially inseminated with semen from a fertile, Dutch-Belted buck (Foote, Hafs, Staples, Gregoire & Bratton, 1963). Those donors yielding blastocysts to be cultured were killed by cervical dislocation 120 hr after LH injection; those yielding blastocysts for control transfers were killed following the culture period, shortly before transfer.

The basic medium was F₁₀, as modified by Daniel (1965), purchased from the Colorado Serum Co., Denver, Colorado. For some of this work rabbit serum was added to constitute either 5% or 10% of the final medium used. The blood used for serum preparation was taken by heart puncture from rabbits on the 5th day of pregnancy and was sterilized by millipore filtration before storage at -15° C. If serum was not included in the medium the blastocysts used were rinsed with some of the medium before culture to eliminate as much carry-over of fluid and debris from the uteri as possible. Except for some of the first 24-hr cultures, penicillin (100 units/ml of medium) and streptomycin (50 µg/ml of medium) were routinely added immediately before culture, in 0.1 ml of saline/20 ml of culture medium.

The 5-day blastocysts were flushed through the cervixes with 2 ml of medium, counted and measured by means of a stereoscopic microscope fitted with an ocular micrometer. For culture at 37° C the blastocysts were retained in 1.5×4.5 mm vials containing 1 ml of medium. Before putting on the screw-cap each vial was gassed with CO₂:air mixture (5:95). Each vial was then put into a light-tight 2.5×6.0 mm screw-capped bottle, which was similarly gassed, and finally sealed with masking tape. About 20 min elapsed between the death of a donor and the start of the culture period. Just before the end of the predetermined culture period, the recipients were anaesthetized with pentobarbital sodium (P.B.S., Bioceutic Laboratories) and laparotomized. It was verified by inspection of the ovaries that ovulation had taken place and corpora lutea were present. Control blastocysts were obtained and handled utilizing the procedure described above. The blastocysts were removed from culture, measured and transferred to the left uterine horn of the recipient; the control blastocysts were transferred to the right uterine horn of the same recipient. Blastocysts were handled in sterilized capillary tubes and transferred with minimal transfer of fluids by slow ejection into the uterine lumen through a stab wound in the uterine wall near the ovarian end. No more than five blastocysts were transferred to a single horn.

On Day 12 of gestation all recipients were laparotomized and the number, size, position and appearance of implantation sites were recorded. The recipients were subsequently killed by cervical dislocation on Day 27, and the viability, number, position and weight of foetuses were noted and each was inspected for the presence of gross malformations. All were anaesthetized, fixed in 70% ethanol, and processed for skeletal examination (Staples & Schnell, 1964).

Blastocysts were initially cultured at 37° C for 24 hr. During culture without serum, many blastocysts collapsed (52/145) but upon addition of 5% serum significantly fewer ($P<0.01$) collapsed (10/61). The non-collapsed blastocysts appeared normal under the dissecting microscope, in that the surface cellular arrangement appeared to be uniform, but the diameters of these blastocysts were only $1.64\text{ mm} \pm 0.05$ upon culture without serum, and $1.69\text{ mm} \pm 0.08$ with serum included against $3.41\text{ mm} \pm 0.05$ among 142 control blastocysts. Fifty-eight of these intact blastocysts were then transferred to pseudopregnant does, but viable foetuses were not obtained at autopsy on Day 27 of gestation. In fact, only five implantation sites were observed upon laparotomy on Day 12 and the diameter of these was about one-fourth the normal size.

In view of this negative response, blastocysts were next cultured for 8 hr. The addition of serum was found necessary to prevent blastocyst collapse (0/64 against 17/49) and to obtain an increase in size comparable to that obtained *in vivo* (Table 1). Blastocyst viability (Table 2) did not appear to be adversely affected by culture at 37° C for 8 hr provided serum was added to the modified F₁₀ medium.

Following culture for 16 hr, a few blastocysts collapsed (6/64) in spite of the presence of serum and although the blastocysts continued to increase in size, they did so at slightly less than the normal rate *in vivo* (Table 1). It is noteworthy, however, that seven live young were recovered on Day 27, following transfer of twenty-five blastocysts that had been cultured *in vitro* for 16 hr. Percentage viability was less than in controls, but was not significantly different ($0.10 < P < 0.20$) from that obtained after 8 hr of culture.

TABLE 1

BLASTOCYST SIZE BEFORE AND AFTER CULTURE AT 37° C IN MODIFIED F₁₀ MEDIUM*

Hours of culture	Medium	Cultured blastocysts			Control blastocysts	
		No.	Initial (mm ± SE)	Final (mm ± SE)	No.	Size (mm ± SE)
8	No serum	49	1.15 ± 0.05	1.28 ± 0.05	69	1.50 ± 0.05
	Serum (10%)	64	1.37 ± 0.03	1.62 ± 0.05		
16	Serum (10%)	64	1.35 ± 0.03	1.92 ± 0.07	29	2.42 ± 0.10

* Only intact blastocysts included.

TABLE 2

VIABILITY OF CULTURED BLASTOCYSTS UPON TRANSFER TO PSEUDOPREGNANT DOES*

Hours of culture	Medium	No. blastocysts transferred	% Blastocysts resulting in implants (Day 13)	% Blastocysts resulting in live foetuses (Day 27)	No. recipients with live young /group
8	No serum	15	46.7	6.7	1/4
	Serum (10%)	25	52.0	40.0	5/5
	Control	55	63.6	49.1	11/14
16	Serum (10%)	25	60.0	28.0	3/5
	Control	20	75.0	70.0	6/7

* Only intact blastocysts transferred.

Table 2 included all results obtained following transfer of non-cultured blastocysts but results from cultured blastocysts were included only from those does with at least one viable foetus on the control side, the argument being that if control blastocysts failed to survive in a given doe, the experimental or cultured blastocysts in the contralateral horn could not be expected to do so, and, if the cultured blastocysts did not develop either, failure could not be attributed solely to the effects of culture. In fact, cultured blastocysts never survived if control blastocysts failed in the contralateral horn. If the data on all cultured blastocysts are included, regardless of the result in the contralateral

control horn, then after an 8 hr culture period with serum, 11/50 blastocysts developed to Day 27 compared with 1/25 in the absence of serum. The results after 16 hr of culture remain unchanged.

On gross examination of the young obtained on Day 27 of gestation as well as examination of the cleared skeletons, all appeared to be normal.

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